Investigating the effects of ebselen, a potential new lithium mimetic, on glutamate transmission

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Introduction: Ebselen was originally developed as a glutathione peroxidase mimic in the treatment of stroke (Parnham & Sies, 2013), and recently explored as a safer alternative to lithium in the treatment of bipolar disorder (Singh et al., 2013). Coincidentally, using magnetic resonance spectroscopy (MRS) we discovered that ebselen reduces glutamate and Glx (glutamate+glutamine) in the anterior cingulate cortex of healthy volunteers (Masaki et al., 2016), consistent with it being an inhibitor of glutaminase enzyme (Thomas et al., 2013). Glutamate is the major excitatory neurotransmitter in the CNS (see Figure 1 for a schematic illustration of the glutamatergic synapse) and is implicated in several psychiatric diseases including bipolar disorder (Jun et al., 2014). Accordingly, several MRS studies in bipolar patients have shown an elevation of Glx in the brain (Dager et al., 2004; Gigante et al., 2012; Yildiz-Yesiloglu & Ankerst, 2006). However, MRS cannot distinguish between 'functional' glutamate in the synapse and stored metabolic glutamate, the former likely being more therapeutically relevant. Therefore, it is important to determine if ebselen's ability to decrease glutamate is reflected in altered glutamatergic neurotransmission or is a consequence of metabolism alone.

Changes in glutamatergic transmission may be quantifiable using positron emission tomography (PET) and radiotracers targeting the metabotropic glutamate receptor 5 (mGluR5). Several radiotracers targeting mGluR5 are negative allosteric modulators of the receptor and therefore altered glutamate at the orthosteric site would affect the ability of the radiotracer to bind to the allosteric site. For example, elevated glutamate in the synapse should decrease tracer binding and vice versa. Most studies have used the mGluR5 radiotracer, (3-(6-methyl-pyridin-2-ylethynyl)-cyclohex-2-enone-*O*-^{11}C-methyl-oxime) ([^{11}C]ABP688) (Ametamey et al., 2006) to demonstrate acute changes in glutamate transmission.

Two such studies in non-human primates showed that [^{11}C]ABP688 uptake was reduced on administration of an acute *N*-acetylcysteine challenge, which is expected to increase extracellular glutamate *via* activation of the cystine-glutamate transporter (Miyake et al., 2011; Sandiego et al., 2013). The first study (Miyake et al., 2011) showed a global reduction in the radiotracer binding potential of approximately 17%, likely due to *N*-acetylcysteine increasing glutamate levels, as expected. The second study (Sandiego et al., 2013) showed a similar effect, with added confirmation of dose-dependence. However, these results were confounded by high test-retest variability and

did not reach statistical significance. The authors proposed that another radiotracer, ^{18}F-3-fluoro-5-[(pyridin-3-yl)ethynyl]benzonitrile ([^{18}F]FPEB), which has a better test-retest reliability (Kuwabara et al., 2011), might be a more suitable probe of cerebral glutamatergic transmission (Sandiego et al., 2013).

In this study, we tested the hypothesis that ebselen treatment increases [^{18}F]FPEB brain uptake compared to vehicle treatment in rats. We reasoned that ebselen, by inhibiting glutaminase, would reduce glutamate transmission and thereby increase [^{18}F]FPEB uptake.

Methods: All animal experiments were conducted in accordance with the Home Office Animals (Scientific procedures) Act, UK, 1986 and were approved by the King's College London ethical review committee. Ebselen was prepared as described (Singh et al., 2013) by mixing and vortexing of small volumes of 0.5 M ebselen in DMSO with 40% *w/v* of hydropropyl beta-cyclodextrin, until the final volume was made up with 4% *w/v* hydropropyl beta-cyclodextrin. The vehicle was prepared in the same way with the exclusion of ebselen. The experiments were carried out over a period of 3 days and were started at 1 pm ± 1 h. Total of eight adult Sprague-Dawley rats were randomised to receive either ebselen (5 mg/kg, n=4) or vehicle (n=4). Isoflurane anaesthesia was induced (5% in oxygen) and maintained at 2%, flow rate of 1 mL/min. The tail vein was cannulated and either ebselen or vehicle administered intravenously (IV) 15 minutes prior to [^{18}F]FPEB, through the same canula.

Scanning was carried out using a BioScan nanoPET-CT^{Plus} (Mediso, Hungary) scanner and images were acquired over 45 min from [^{18}F]FPEB injection. The scans were obtained at 400–600 keV energy window, 5 ns coincidence time and coincidence mode of 1–5. CT scan was performed at standard frame resolution (512 × 512 pixels), 55 kVP tube voltage, 600 ms of exposure time and 360° projections. Reconstruction was carried out using ordered subset expectation maximisation (OSEM) iterative reconstruction algorithm (propriety software, Mediso Ltd.). Corrections for decay, randoms, crystal dead time, detector normalisation and attenuation correction were implemented. Images were reconstructed with a voxel size of $0.25\times0.25\times0.25 mm^3$ for CT, and $0.4\times0.4\times0.4 mm^3$ for PET. The PET and CT images were co-registered automatically. Images were analysed using VivoQuant 2.0 (Invicro LLC) software. The 3D rat brain atlas template was used to determine brain region (9 pre-selected regions including cerebellum) specific radiotracer concentrations. PET data were analysed using Logan graphical analysis with cerebellum as normative region and distribution volume ratio (DVR) as the main parameter of interest, as previously reported (Abdallah et al., 2017; Miyake et al., 2011).

The radioligand binding assay for mGluR5 was carried out by Eurofins Discovery Services, France, to assess if ebselen interfered directly with glutamate binding. Briefly, varying concentrations of ebselen and 40 nM

[^{3}H]quisqualate (which binds to the mGluR5 orthosteric site) were incubated for 120 min with a cellular homogenate preparation containing mGluR5 receptors, and then filtered to separate bound from free tracer. Results are expressed as a percentage inhibition of control binding (40 nM [^{3}H]quisqualate in absence of ebselen), and an inhibition greater than 50% was considered significant.

[^{18}F]FPEB was synthesised as outlined by Sullivan et al. (2013). There were no significant differences in molar activity (A_m, GBq/µmol)(Coenen et al., 2017), injected dose, and weight between the two groups of rats. Significance was tested using two-way analysis of variance (ANOVA) and Holm-Sidak correction for multiple comparisons. Where appropriate, unpaired t-tests were performed to compare two means. A 'p' value of less than 0.05 was considered significant.

Results: There was a statistically significant effect of ebselen treatment ($F(1,48) = 5.112$, $p = 0.028$) and a global increase (~8%) in [^{18}F]FPEB brain uptake overall, although no single region showed statistical significance (Fig. 2). All 8 regions showed an increase in DVR, with the olfactory region and the amygdala showing the largest increases of 18.6% and 15.5%, respectively, and the midbrain the smallest of 2.4%. Importantly, we also confirmed that the cerebellar uptake (measured as SUV) of [^{18}F]FPEB showed no significant differences ($p = 0.44$) between vehicle and ebselen treated rats, which validated its suitability as a normative region in this study. Please see Fig. 5A-C showing the SUV time-activity curves for the two highest binding regions, the olfactory bulb and amygdala, and the normative region (cerebellum).

We additionally verified that ebselen does not directly interfere with glutamate binding at the orthosteric site of mGluR5, as this might lead to changes observed not due to glutamate in the synapse, but rather due to ebselen blocking the mGluR5 itself. Ebselen had no effect on the binding of [^{3}H]quisqualate to mGluR5 (Fig. 3), demonstrating that ebselen does not directly bind to the receptor's orthosteric site.

Discussion: In this pilot study we have demonstrated, for the first time, that acute administration of ebselen potentially decreases synaptic glutamate levels, as measured by an increased brain uptake of [^{18}F]FPEB, an mGluR5 radiotracer. Additionally, we have also demonstrated that [^{18}F]FPEB is potentially a suitable tool to image glutamate transmitter changes. To the best of our knowledge, this is the first study to use [^{18}F]FPEB for measuring acute changes in glutamate transmission although a similar radiotracer [^{11}C]ABP688 has been used previously in non-human studies (DeLorenzo et al., 2015; Miyake et al., 2011; R Zimmer et al., 2015). This is of significance as glutamate, being the major excitatory neurotransmitter in the brain, has wide implications in diseases ranging from

autism to neurodegeneration and furthering this work to validate the mGluR5 tracers in the clinic would be crucial for our understanding of glutamate in disease and treatment.

Although the results shown here are statistically significant, one caveat is that the low sample number may provide insufficient power to detect region-specific changes. However, our results are consistent with the other studies where changes were observed across all brain regions, but no single region showed statistical significance after an acute challenge (Miyake et al., 2011; Sandiego et al., 2013). Additional acute studies of ebselen investigating the effects of dose and time, including infusion rather than bolus administration, and sequence of tracer and drug administration, on the response are necessary and would be revealing. As an example, the Logan method assumes linearity of the intercept when the distribution volume in the target and reference tissues are at equilibrium, and ebselen administration in this experiment may have perturbed the equilibrium. We tested for the quality of the Logan regression in the two conditions by using the residual sum of squares (RSS) as performance metric and there was no significant different between the ebselen and vehicle treated conditions.

In other pilot studies using prolonged dosing with ebselen (results not shown) we observed a significant ($p<0.001$) positive correlation between the weight of animal from baseline to two weeks and an increased [^{18}F]FPEB uptake. Therefore, we believe that it is critical for any longitudinal study to consider the effects of animal age and weight to separate out the pharmacological from the physiological effects.

The use of the cerebellum as a normative region should also be tested against an arterial input comparator in future studies. Although similar studies in non-human primates (Miyake et al., 2011; Sandiego et al., 2013) and rats (Elmenhorst et al., 2010) have used the cerebellum as the reference region with success, it also been demonstrated in humans that the cerebellum has 60% specific binding of mGlur5 radiotracers (Lohith et al., 2017), in spite of being the region with the lowest uptake. In our experiments, if we assume that there was an increase in [^{18}F]FPEB binding in the cerebellum in ebselen treated rats, compared to vehicle, we would observe a decreased DVR response in all other regions, i.e., we would be underestimating our results. Thus, although sub-optimal, we believe that the use of the cerebellum, in this instance, is correct.

It is also important to point out that preclinical PET imaging experiments, especially those measuring glutamate, may be confounded by the effects of anaesthesia. For example, a study in healthy conscious people demonstrated the ability of [^{11}C]ABP688 PET to detect glutamatergic changes upon administration of ketamine

(DeLorenzo et al., 2015) but another study could not replicate these effects in anaesthetised rats (Kosten et al., 2018). Given glutamate toxicity, the levels of its release at synapses are likely to be tightly controlled. If general anaesthesia indeed dampens synaptic glutamate release, this will additionally limit the power to detect glutamate transmission fluctuations in preclinical studies using anaesthetised rodents. It would also be worthwhile testing the effects of anaesthesia and comparing glutamatergic transmission with and without anaesthesia.

In this study, we have suggested that the effects of decreased glutamate might be mediated through inhibition of glutaminase by ebselen; however, it is recognised that ebselen has rich pharmacology. Indeed, ebselen also inhibits glutamate dehydrogenase (Zhu et al., 2017) and it is feasible to extrapolate that the overall effects of ebselen are likely due to a summation of its effects on multiple targets.

Conclusions: Acute administration of ebselen decreased synaptic glutamate transmission as demonstrated by increased uptake of [^{18}F]FPEB. Further experiments need to be conducted to ascertain ebselen's dose-related effects on synaptic glutamate *in vivo*. Additionally, [^{18}F]FPEB appears to be a useful radiotracer to investigate glutamatergic transmission *in vivo* which would be critical in furthering our understanding of glutamatergic transmission and the pharmacological influences over it.

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Disclosures: NS holds a method of use patent granted on the use of ebselen for the treatment of bipolar disorder (US20140094449A1). All other authors declare that they have no conflicts of interest.

Data sharing: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figures:

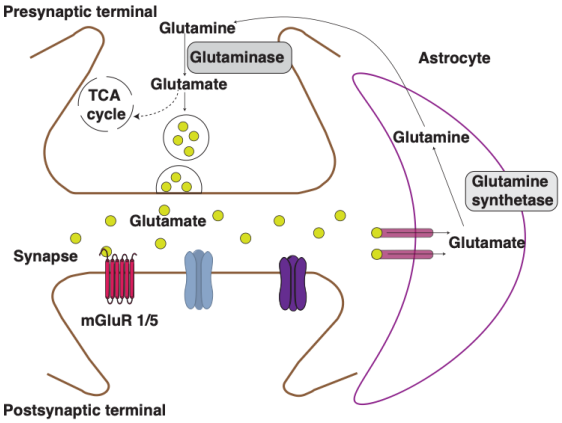

Figure 1. Schematic representation of the pre and post synaptic neurons and an astrocyte showing glutamate release, reuptake and recycling.

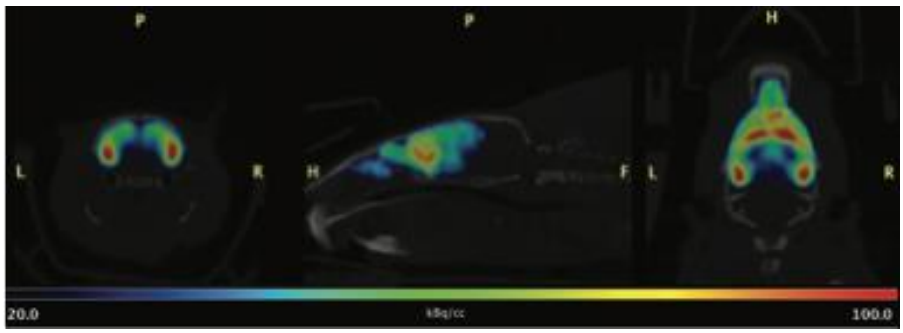

Figure 1. Representative images (coronal, sagittal, and axial) showing the uptake of [^{18}F]FPEB in the rat brain. (Images constructed using PMOD 3.8).

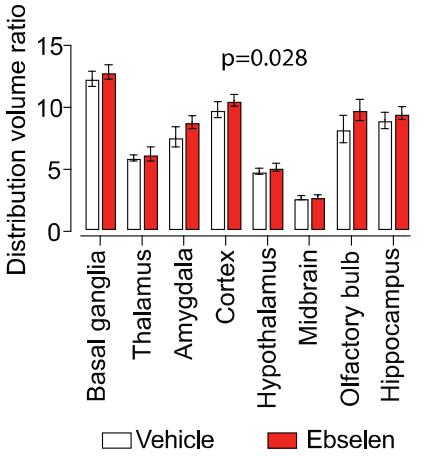

Figure 3. Bar graphs showing the distribution volume ratio (DVR) of [^{18}F]FPEB on the y-axis (mean ± SEM) across 8 brain regions on the x-axis. Two-way ANOVA testing showed a significant effect of treatment (p = 0.028)

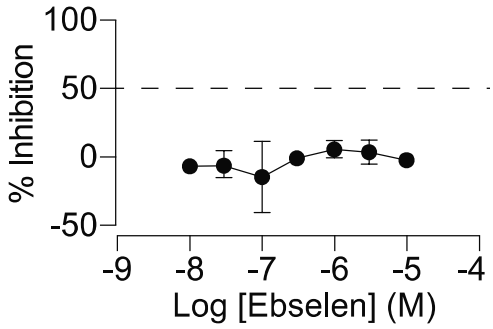

Figure 4. Dose response plot showing the effect of ebselen on [^{3}H]quisqualate binding to the mGluR5 orthosteric site (mean ± SD). Significant inhibition would be achieved above the dotted line showing 50% inhibition. Ebselen did not inhibit [^{3}H]quisqualate binding.

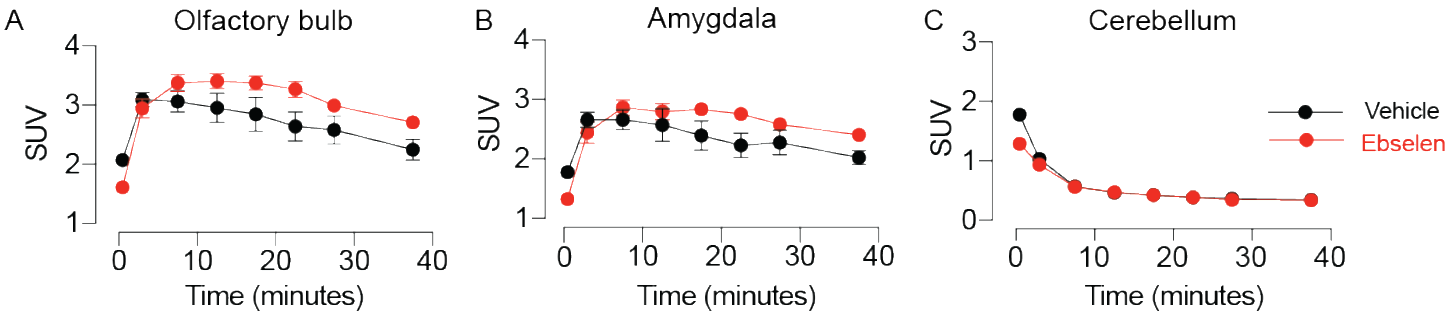

Figure 5. Time activity curves after ebselen (in red) or vehicle (in black) treated rats for the (A) olfactory bulb, (B) amygdala and, (C) cerebellum. The standardised uptake value (SUV) is shown on the y-axis mean ± SEM) and time in minutes on the x-axis.

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